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The nascent polypeptide-associated complex (NAC) promotes interaction of ribosomes with the mitochondrial surface in vivo

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Abstract The nascent polypeptide-associated complex (NAC) is a peripheral component of cytoplasmic ribosomes, and interacts with nascent chains as they leave the ribosome. Yeast mutants lacking NAC translate polypeptides normally, but have fewer ribosomes associated with the mitochondrial surface. The mutants lacking NAC suffer mitochondrial defects and have decreased levels of proteins like fumarase, normally targeted to mitochondria co-translationally. NAC might contribute to a ribosomal environment in which amino-terminal, mitochondrial targeting sequences can effectively adopt their appropriate conformation.

Key words: Ribosome; Mitochondrion; Protein targeting; Nascent chain

1. Introduction

In eukaryotic cells, many proteins made in the cytosol need to be targeted to other subcellular compartments. Ribosomes can become associated with membranes in the course of translating polypeptides destined to leave the cytosol. For secreted proteins, this co-translational association is tightly coupled through the action of the signal recognition particle (SRP) [1]. SRP binds the amino-terminal signal sequence of a polypeptide destined for secretion as it emerges from the ribosome, and inhibits further translation until the ribosome docks with the endoplasmic reticulum [2,3]. Even in the absence of a nascent polypeptide, ribosomes can bind the endoplasmic reticulum through an intrinsic affinity for the translocon [4–11]. Interaction of ribosomes with the translocon is largely mediated through the surface features of the 28S rRNA [12] and cryo-electron microscopy reveals that once bound, the polypeptide exit channel in the ribosome aligns with the central channel of the translocon [11].

Most mitochondrial proteins are also translated on cytoplasmic ribosomes [13–15]. While many of these complete synthesis on cytosolic ribosomes to be imported post-translationally into mitochondria, ribosomes programmed by mRNA encoding mitochondrial precursor proteins also accumulate on the surface of mitochondria and remain translationally active [16–19]. There is no evidence for the existence of a mitochondrial equivalent to SRP. Instead, mitochondrial membrane-bound polysome complexes probably represent those that have continued to translate after a partly made precursor protein interacts with the translocase in the outer mitochondrial membrane (TOM complex), where all subsequent precursor proteins synthesized from that polysome would be imported co-translationally [15,20].

The nascent polypeptide-associated complex (NAC) was identified as a heterodimeric factor in mammalian cell extracts that associated with nascent chains including those destined for the endoplasmic reticulum and mitochondria [21]. It has been suggested that NAC provides a protective environment for short nascent polypeptide segments [22,23]. However, NAC has also been found in the nucleus of mammalian cells where it functions in transcriptional control [24], and the yeast homolog of NAC, the Egd1/Egd2 complex, was first identified as a factor that can bind DNA fragments representing transcriptional control elements [25].

Yeast mutants lacking NAC have slight defects in growth rate and cell morphology consistent with defects in protein targeting [26,27], and in vitro studies show that NAC can stimulate import of nascent precursor proteins into isolated mitochondria [23]. Here we show that that the Egd1p/Egd2p complex (NAC) is a peripheral component of cytoplasmic ribosomes, including membrane-associated ribosomes that have not been salt-stripped. Further, NAC is required to promote interaction of ribosomes with the mitochondrial surface in vivo: yeast mutants lacking NAC have less than 10% of wild-type levels of mitochondria-bound ribosomes and have a defect in respiratory function. NAC function is required to maintain efficient targeting of some mitochondrial proteins, including fumarase, malate dehydrogenase and the genome maintenance factor Mmf1p.

2. Materials and methods

2.1. Yeast strains

To create a null mutant lacking NAC, a fragment of the *EGD1* gene was digested with *Hin*dIII, and a *Hin*dIII fragment of the *URA3* gene was inserted disrupting *EGD1* after the codon corresponding to Arg_{25}. The *egd1*::*URA3* fragment was transformed into the yeast strains JK9-3d**a** and YRLG2 [24] to generate the Δ*egd1* strain YRLG17 (*Mat***a**, *ura3*, *leu2*, *his4*, *trp1*, *egd1*::*URA3*) and the Δ*egd1*, Δ*egd2* strain YRLG16 (*Mat***a**, *ura3*, *leu2*, *his3*, *trp1*, *ade2*, *egd2*::*ADE2*, *egd1*::*URA3*) respectively. Disruption of the *EGD1* gene was confirmed by Southern analysis.

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Abbreviations: SRP, signal recognition particle; TOM complex, translocase in the outer mitochondrial membrane; NAC, nascent polypeptide-associated complex; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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2.2. Subcellular fractionation

Mitochondria were isolated [28] and purified from contaminating nuclei and microsomal membranes on Nycodenz gradients [29]. To stabilize ribosome association, mitochondria were prepared [30] by homogenization in BB6.0 buffer (20 mM 2-[*N*-morpholino]ethanesulfonic acid, 0.6 M sorbitol, pH 6.0) including 2 mM $MgCl_2$ and this crude preparation of mitochondria further purified on Nycodenz gradients containing 2 mM $MgCl_2$.

To isolate microsomal membranes free of mitochondrial contamination, spheroplasts were homogenized in BB6.0 containing 2 mM $MgCl_2$ with 15 strokes of a loose-fitting Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation at 5000 rpm for 5 min in an SS34 rotor (Sorvall), and the supernatant centrifuged at 10 000 rpm for 10 min (SS34 rotor, Sorvall) to pellet the mitochondria, nuclear membranes and some endoplasmic reticulum. The supernatant was centrifuged for 30 min at 20 000 rpm (SS34 rotor, Sorvall). Pellets were washed once in 10 mM Tris–HCl, 2 mM $MgCl_2$ (pH 7.4) and recentrifuged to yield crude microsomes.

Yeast cytosol, ribosomes and polysomes were purified as previously described [31,32]. Linear (15–50%) sucrose gradients prepared in RB buffer (20 mM HEPES pH 7.4, 5 mM $MgCl_2$, 10 mM KCl, 2 mM EGTA) were fractionated on an ISCO Model 640 density gradient fractionator and recorded on an ISCO Model UA-5 absorbance monitor. Fractions (1.2 ml) were collected, and proteins precipitated with 10% trichloroacetic acid for analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting.

To monitor translation in vivo, cells were grown to an OD_{600} of 0.5 U, resuspended in fresh semisynthetic medium containing ^{35}S-translabel (25 Ci/OD unit) for 10 min. The labeled cells were reisolated and resuspended in TE lysis buffer (20 mM HEPES pH 7.4, 100 mM potassium acetate pH 7.4, 2 mM magnesium acetate, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1% Triton X-100) and broken with glass beads for two 1.5 min bursts, with 5 min incubation on ice in between. Cell debris was removed by centrifugation at $15\,000 \times g$ for 10 min at 4°C in a bench top centrifuge (Heraeus) and the supernatant analyzed by immunoprecipitation [33].

To determine respiratory competence, wild-type and Δ*egd1*, Δ*egd2* cells were grown in rich medium containing glucose as a carbon source (YPAD) for 6 h ($OD_{600} < 1.5$), harvested and resuspended in water ($OD_{600} = 1.0$). Serial dilutions (1:5) were plated onto YPAD (rich medium with 3% glucose as the carbon source) and YPEG (rich medium with 3% ethanol+3% glycerol as carbon sources) plates and incubated for 3 days at 30°C. Colonies formed were counted: 100% of wild-type cells, but only 58% of Δ*egd1*, Δ*egd2* cells, could form colonies on YPEG (average of three independent experiments).

2.3. Miscellaneous

A protein of approximately 14 kDa showed decreased levels in mitochondria isolated from Δ*egd1*, Δ*egd2* cells, and amino-terminal sequencing of proteins in this region of the gel suggested the protein was Mmf1p (from the processed amino-terminal sequence LTPVSTKLAPPAA). Hexahistidine-tagged Egd1p and hexahistidine-tagged Mmf1p were expressed in *Escherichia coli* and purified using Ni-NTA resin (Qiagen), polyclonal antibodies were raised in rabbits immunized with either of the purified proteins. SDS–PAGE, immunoblotting and blotting to polyvinylidene difluoride membranes for amino-terminal sequencing were as previously described [33].

3. Results and discussion

When yeast cell extracts are fractionated by ultracentrifugation, NAC is found in the $100\,000 \times g$ pellet fraction [26,27]. Sucrose density gradient centrifugation of extracts prepared in buffers of low ionic strength shows there is little or no free NAC in the cytosol (Fig. 1A, lanes 1 and 2), with the majority of the protein co-purifying with cytoplasmic ribosomes (lanes 4–7). Yeast proteome analysis has revealed that the levels of the two subunits of NAC (Egd1p and Egd2p) are in approximate stoichiometry with that of core ribosomal proteins [26], suggesting one NAC heterodimer per ribosome.

Electron microscopy has revealed a proportion of cellular ribosomes attached to the mitochondrial outer membrane [34,35], and mitochondria can be isolated from yeast with translationally active ribosomes still attached to the organelle [30,36]. Semi-quantitative immunoblotting suggests that mitochondria isolated from wild-type yeast cells carry approximately 1% of the ribosomes present in an equivalent amount of total cell extract (Fig. 1B). Yeast cells lacking NAC have a significant decrease in ribosomes bound to the mitochondrial surface: with 25 μg of mitochondrial protein loaded for SDS–PAGE, immunoblotting fails to detect any ribosomal protein L3 in the sample isolated from Δ*egd1*, Δ*egd2* yeast cells (Fig.

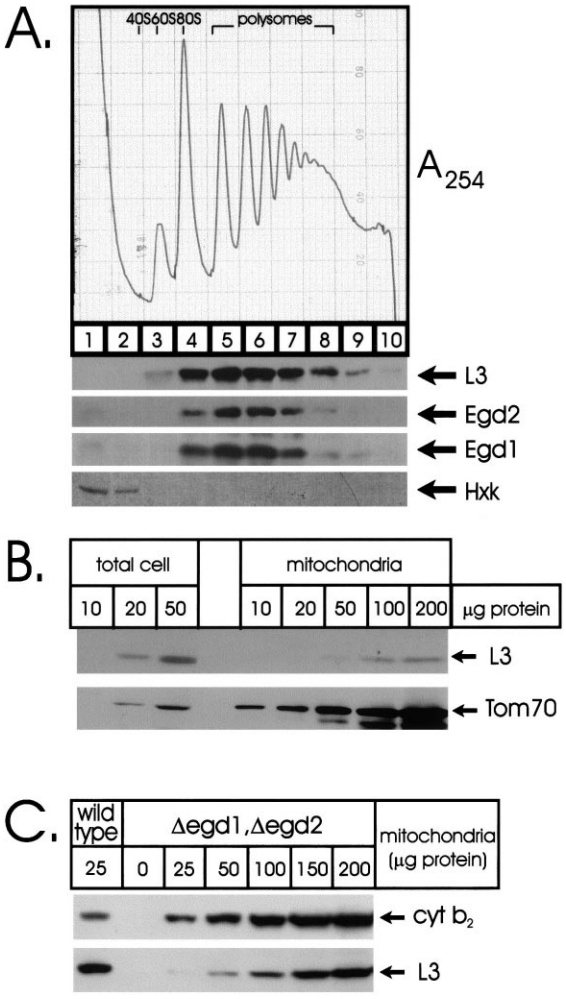

Fig. 1. Yeast NAC is associated with ribosomes, with little or no free NAC in the cytoplasm. A: Cytosol, 40S and 60S subunits, 80S monosomes and polysomes from wild-type yeast cells were separated by sucrose density gradient centrifugation. The gradients were fractionated from top (fraction 1) to bottom (fraction 10) and absorbance was monitored at 254 nm. Fractions were analyzed by SDS–PAGE and immunoblotting with antibodies against Egd2p, Egd1p, hexokinase (Hxk) and the ribosomal protein rpL3. B: Total cell extracts and Nycodenz-purified mitochondria were prepared from wild-type cells and the indicated amounts loaded for SDS–PAGE and immunoblot analysis. Mitochondria are enriched 10–20-fold during purification (from comparison of the signal for Tom70), whereas ribosomes are depleted 10-fold (from comparison of the signal for ribosomal protein L3). C: Mitochondria prepared from wild-type (25 μg mitochondrial protein) and Δ*egd1*, Δ*egd2* cells (25–200 μg of mitochondrial protein as indicated) were analyzed by SDS–PAGE and immunoblotting using antibodies against mitochondrial cytochrome b_2 (cyt b_2) and ribosomal protein L3.

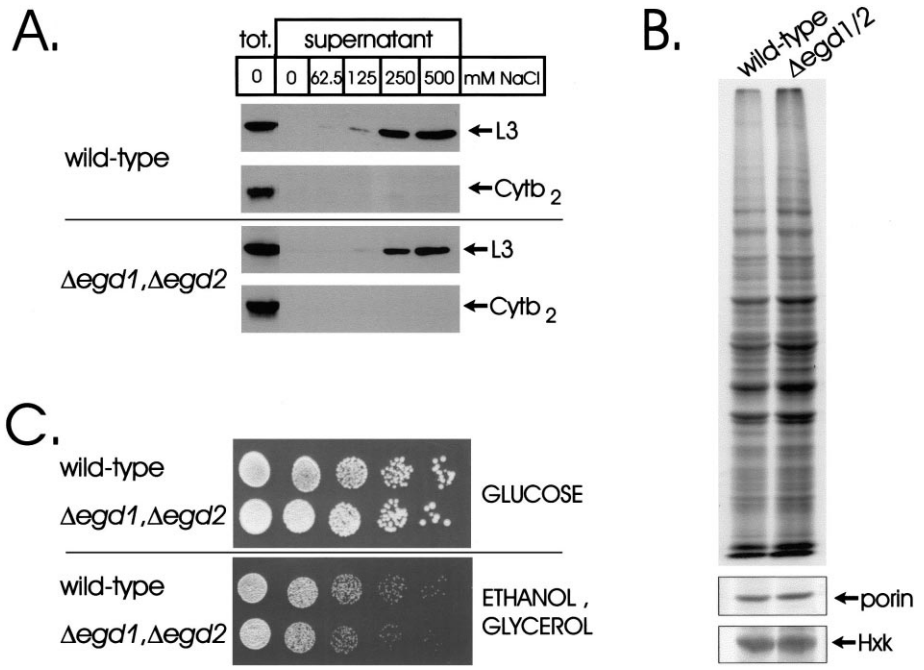

Fig. 2. Ribosomes lacking NAC are translation-competent and can dock to mitochondrial membranes. A: Mitochondria were prepared from either wild-type or Δ*egd1*, Δ*egd2* cells and aliquots washed with buffer containing NaCl. Mitochondria were reisolated, and ribosomes and salt-extractable proteins (supernatant) separated by SDS–PAGE using samples of 50 μg mitochondrial protein (wild-type) or 150 μg mitochondrial protein Δ*egd1*, Δ*egd2* cells) and analyzed by immunoblotting against ribosomal protein L3 and mitochondrial cytochrome b_2 (cyt b_2). B: Wild-type and Δ*egd1*, Δ*egd2* cells were labeled with [^{35}S]methionine, and the soluble protein fraction analyzed by SDS–PAGE (upper panel) or subject to immunoprecipitation with antisera to porin or hexokinase (lower panel). C: Wild-type and Δ*egd1*, Δ*egd2* cells were grown in rich medium containing glucose as a carbon source (YPAD) for 6 h, harvested, resuspended and serial dilutions plated onto YPAD ('glucose') and YPEG ('ethanol, glycerol') plates. Yeast colonies were counted after incubation at 30°C.

1C). Less than 15% of wild-type levels of ribosomes are bound; 8–10 times as much mitochondria must be loaded for L3 to be detected in the mutant extracts at levels approaching that associated with wild-type cells.

One possible explanation is that ribosomes are more easily lost during the isolation procedure in the absence of NAC. However, Fig. 2A shows the salt sensitivity of ribosomes bound to mitochondria isolated from either wild-type or Δ*egd1*, Δ*egd2* cells: 250 mM sodium chloride sufficient to remove half the membrane-bound ribosomes and 500 mM sodium chloride sufficient to completely remove wild-type ribosomes from the mitochondrial surface (upper panel). Similarly, in the absence of NAC 500 mM salt is needed to remove more than half the membrane-bound ribosomes (Fig. 2A, lower panel).

The relative kinetics of translation and import are important considerations dictating ribosome association with the mitochondrial surface [15], but Δ*egd1*, Δ*egd2* yeast cells do not have gross defects in translation. Cells lacking NAC are viable over a broad temperature range with only a slight defect in growth rates [26,27], and there is no effect on the polysome profiles of Δ*egd1*, Δ*egd2* yeast cells (data not shown). This suggests that NAC is not required to maintain normal rates of polypeptide synthesis, since the proportion of polysomes to ribosomal subunits is a measure of translational efficiency. Further, in vivo labeling of cells with ^{35}S revealed no obvious difference in the polypeptide pattern synthesized by wild-type or Δ*egd1*, Δ*egd2* yeast cells (Fig. 2B), and immunoprecipitation analysis of the labeled polypeptides shows no difference in the synthesis of the cytosolic protein hexokinase or the mitochondrial protein porin (Fig. 2B). Like Δ*egd2* cells [26], mutant Δ*egd1*, Δ*egd2* yeast cells accumulate defects in mitochondrial function, with 58% of the cells in log phase growing cultures unable to form colonies on media with the non-fermentative carbon sources ethanol and glycerol (Fig. 2C).

There is no evidence that translation and protein import into mitochondria is obligatorily coupled in vivo [13–15]. All of the work done to date with in vitro assays and the few studies undertaken in vivo suggest that any precursor protein can be imported to some extent into mitochondria post-translationally. However, some precursor proteins can be imported

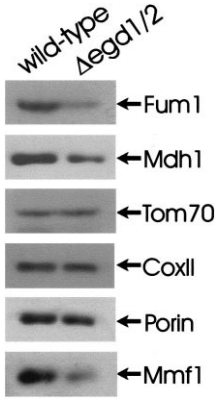

Fig. 3. Mitochondria from or Δ*egd1*, Δ*egd2* cells show selective loss of some mitochondrial proteins. Mitochondria from wild-type and Δ*egd1*, Δ*egd2* cells were isolated and analyzed by SDS–PAGE. Resident levels of specific proteins were determined by immunoblotting against fumarase (Fum1), malate dehydrogenase (Mdh1), Tom70p, cytochrome oxidase subunit II (CoxII), porin and Mmf1p.

more efficiently into mitochondria co-translationally. While SDS–PAGE analysis suggests the vast majority of mitochondrial proteins have no decrease in steady-state levels in the Δ*egd1*, Δ*egd2* yeast cells (data not shown), immunoblotting for a select set of proteins revealed that the level of Fum1p is about 30% in mitochondria from Δ*egd1*, Δ*egd2* cells, Mdh1p about 50% and Mmf1p about 20% (Fig. 3).

Fum1p is an enzyme found in both the mitochondrial matrix and the cytosol of yeast cells, and its relative distribution is maintained by the efficiency of import of the Fum1p precursor protein: after release from free ribosomes, a large proportion of the polypeptide folds too tightly to be translocated readily across the mitochondrial membranes, and processing of these molecules allows their release into the cytosolic pool of enzyme [37,38]. The mitochondrial population of Fum1p is accumulated by co-translational import [38]. The precursor form of malate dehydrogenase (Mdh1p) is also imported efficiently in a co-translational reaction, and import of ribosome-associated Mdh1p nascent chain into isolated mitochondria is entirely dependent on NAC [23]. Mmf1p is required for maintenance of mitochondrial DNA [39], and decreased targeting of Mmf1p to the mitochondria of Δ*egd1*, Δ*egd2* cells might explain the tendency of these mutants to lose mitochondrial DNA.

A defined number of proteinase-sensitive ribosome-binding sites exist on the mitochondrial surface [16,37], and while the number of sites corresponds approximately to the number of TOM complexes present in the outer membrane, the proteins that constitute these ribosome-binding sites have not been identified. However, in studies on the Atm1p precursor Corral-Debrinski et al. showed that one of the key determinants in accumulating polysomes at the mitochondrial surface is found in the first 16 amino acids of the protein sequence [36], and it seems likely that a productive interaction of the presequence with the TOM complex is required to localize ribosomes to mitochondria.

We suggest that NAC is required for effective targeting of ribosome-nascent chain complexes to mitochondria because NAC provides a protective environment for short, amino-terminal nascent chains to adopt appropriate secondary structure. In the case of mitochondrial proteins like Fum1p, Mdh1p, Mmf1p and Atm1p this region incorporates the mitochondrial targeting sequence; NAC might enhance the opportunity for these amino-terminal sequences to adopt an α-helical conformation. This would greatly contribute to the initiation of the import pathway for precursors still in the process of translation [13–15].

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